United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 202

Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type C Beta Antitoxins

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1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine the beta antitoxin content of *Clostridium perfringens* type C antitoxins as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.454. The antitoxin is titrated by a toxin-antitoxin neutralization test using mice as an indicator.

2. Materials

2.1 Equipment

Equivalent equipment may be substituted for any brand name listed below.

2.1.1 Mixer, vortex type

2.1.2 Freezer, -70ºC

2.1.3 Micropipettes, 100-µL and 1000-µL

2.1.4 Refrigerator, 2º- 7ºC

2.1.5 Autoclave

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 *C. perfringens* type C (beta) standard antitoxin IRP 486

2.2.2 *C. perfringens* type C (beta) standard toxin IRP 513(04)

2.2.3 Peptone diluent

2.2.4 Glass screw-cap tubes, 13 x 100-mm

2.2.5 Pipettes, 1-mL, 5-mL, 10-mL, 25-mL

2.2.6 Syringes, 1-cc

2.2.7 Needles, 25- to 27-gauge x 1- to 1 1/4-inch

2.2.8 Screw-top Erlenmeyer flask, 500-mL, with cap

2.2.9 Glass dilution bottles, 160-mL

2.2.10 Polystyrene snap-top tubes, 17 x 100-mm, with caps

2.2.11 Polystyrene screw-cap conical tubes, 17 x 120-mm

2.2.12 Water, distilled or deionized, or water of equivalent purity

2.2.13 Tips for micropipettes

2.3 Test animals

White Swiss nonpregnant female mice, 16-20 g (Five mice are required for each toxin-antitoxin mixture.)

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in the safe handling of clostridial toxins. Personnel need specific training in the care and handling of laboratory mice.

3.2 Preparation of equipment/instrumentation

All equipment is operated according to manufacturers' instructions.

3.3 Preparation of reagents/control procedures

3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water q.s. to	800 mL

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill 500-mL Erlenmeyer flask no more than 3/4 full with diluent.

Autoclave with caps loosened at 121°C for 25 to 30 minutes. Cool flasks and tighten caps. Store diluent at 2°- 7°C for up to 3 months.

3.3.2 Preparation of *C. perfringens* type C standard beta antitoxin

1. *Clostridium perfringens* type C antitoxin IRP 486 contains 500 units of beta antitoxin per mL (AU/mL) and has been standardized against the World Health Organization *C. perfringens* (*C. welchii*) type C International antitoxin. Each vial contains 1.3 mL of antitoxin.

2. A dilution of standard beta antitoxin containing 10 (AU/mL) is used in the toxin-neutralization test. To prepare the dilution, add 1.0 mL of well mixed IRP 486 to 9.0 mL of peptone diluent in a 17 x 100-mm snap-top tube. IRP 486, diluted 1:10, is stable when stored at -70°± 5°C.

3. Further dilute the toxin to 1:50 by adding 1.0 mL of diluted toxin (1:10) to 4.0 mL of diluent in a 17 x 100-mm snap-top tube.

3.3.3 Preparation of *C. perfringens* type C standard beta toxin

1. Prepare a 1:10 dilution of *C. perfringens* type C beta toxin by adding 1.0 mL of IRP 513(04) to 9.0 mL of peptone diluent in a 17 x 100-mm snap-top tube. Dispense diluted toxin in 1.5-mL amounts into 13 x 100-mm screw-cap tubes. IRP 513(04), diluted 1:10, is stable when stored at -70°± 5°C.

2. Further dilute the toxin to 1:110 by adding 1.0 mL of diluted (1:10) toxin to 10.0 mL of peptone diluent in a 17 x 120-mm conical tube. For the purpose of this test, the 1:110 dilution of IRP 513(04) is referred to as the standard beta toxin.

Note: A volume of 0.5 mL of standard beta toxin and 0.5 mL of peptone diluent represents 10 L_o doses. A volume of 0.8 mL of the standard beta toxin and 0.2 mL of peptone diluent represents 10 L_+ doses (see Sections 4.1.1 and 4.1.2). For the purposes of this SAM, 10 L_o dose is defined as the greatest amount of toxin that, when mixed with 10 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.2 mL of this mixture. The 10 L_+ dose is defined as the least amount of toxin that, when mixed with 10 AU, results in the death of 80%-100% of all mice inoculated IV with 0.2 mL of this mixture.

4. Performance of the Test

4.1 Toxin neutralization

4.1.1 Product and standard beta toxin

1. Mix a sufficient volume of standard beta toxin and peptone diluent (0.5 mL of standard beta toxin and 0.5 mL peptone diluent [10 L_o doses]) for each product antitoxin dilution and the L_o control using a 17 x 120-mm conical tube.

2. Add 1 ml of each product antitoxin dilution (see table below) to 1 mL of the standard beta toxin-peptone diluent mixture (10 L_o doses) in 17 x 100-mm snap-top tubes. Mix each tube with a vortex-type mixer.

Int'l AU tested	Unknown Antitoxin	10 L_o doses	
		Std Toxin	Diluent
500	1 mL diluted 1:50 (0.5 mL product + 24.5 mL dil.)	0.5 mL	0.5 mL
600	1 mL diluted 1:60 (0.5 mL product + 29.5 mL dil.)	0.5 mL	0.5 mL
1200	1 mL diluted 1:120 (0.3 mL product + 35.7 mL dil.)	0.5 mL	0.5 mL

3. Let the mixtures sit at 22°- 26°C (room temperature) for 1 hour.

4. Place tubes in ice.

4.1.2 Standard beta toxin and standard beta antitoxin controls

1. Add 1.0 mL of standard beta antitoxin containing 10 AU/mL to 1.0 mL of the standard beta toxin-peptone diluent mixture (10 L_o doses) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

2. Add 1.0 mL of standard beta antitoxin containing 10 AU/mL to 0.8 mL of standard beta toxin and 0.2 mL of peptone diluent (10 L_+ doses) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

3. Let the mixtures stand at 22º- 26°C for 1 hour.

4. Place tubes in ice.

4.2 Inoculation of mice

4.2.1 Inject 0.2 mL of each standard beta toxin-product antitoxin mixture into each of 5 mice.

4.2.2 Inject 0.2 mL of each standard beta toxin-standard beta antitoxin mixture into each of 5 mice.

4.2.3 Inoculate all mice intravenously into a lateral tail vein. Use 1-cc needle locking syringes fitted with 25- to 27-gauge x 1- to 1 1/4-inch needles.

4.2.4 Always inoculate the mice receiving the standard beta toxin-standard beta antitoxin mixtures (controls) **last**.

4.2.5 Mouse inoculations should be completed within 1 hour of placing the toxin-antitoxin mixtures in the ice.

4.2.6 The test is concluded 24 hours after the mice are inoculated.

5. Interpretation of the Test Results

5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 10 L_o/10 AU control mixture must survive.

5.1.2 At least 4 of the 5 mice inoculated with the standard 10 L_+/10 AU control mixture must die.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5.2 Interpretation of serial results

5.2.1 The product contains at least 500 International Units of beta antitoxin per mL if 5 of the 5 mice inoculated with the 1:50 dilution of product-standard beta toxin mixture survive.

5.2.2 The product contains at least 600 International Units of beta antitoxin per mL if 5 of the 5 mice inoculated with the 1:60 dilution of product-standard beta toxin survive.

5.2.3 The product is considered unsatisfactory if it contains less than 500 International Units of beta antitoxin per mL. (If any mice inoculated with the 1:50 dilution and 10 L_o doses of standard beta toxin die, the product is considered to contain less than 500 International Units per mL.)

6. Reporting of Test Results

Report the results of the test(s) as described by standard operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.454, U.S. Government Printing Office, Washington DC.

7.2 History of toxin: *C. perfringens* type C culture #4414, used to produce IRP 513(04), and was obtained from Coopers Animal Health, Inc., 1201 Douglas Avenue, Kansas City, Kansas, on July 28, 1975. The number of passages is unknown.

7.3 History of antitoxin*: C. perfringens* type C (beta) antitoxin IRP 486 was produced in goats hyperimmunized with multiple injections of purified *C. perfringens* type C toxoid and toxin during a 6 month period.

8. Summary of Revisions

Version .03

- The document number has been changed from BBSAM0202 to SAM 202.
- **3.3.3:** The standard beta toxin use dilution has been adjusted.

Version .02

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- IRP 119 has changed to IRP 486 throughout the document.
- IRP 418 has changed to IRP 513(04) throughout the document.

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- **4.1** The format and content have been modified to clarify the L_O and L_+ levels of the Toxin Neutralization process.

- Humane endpoint language has been added.

- Dilution/holding vessel sizes have been added for clarification.

- The contact person has been changed to Janet M. Wilson